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Diet Affects Immunologically Relevant Gene Expression in Brains of Toxoplasma gondii Infected

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Diet Affects Immunologically Relevant Gene Expression in Brains of *Toxoplasma gondii* Infected Mice

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By

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Table of Contents

List of Tables

List of Figures

Abstract

The protozoan parasite, *Toxoplasma gondii*, is a serious health concern for pregnant women and HIV/AIDS individuals. Previous studies provided evidence that dietary supplementation with the antioxidants vitamin E and selenium is harmful to infected mice; however, a diet lacking in these antioxidants resulted in reduced pathology, including decreased counts of tissue cysts. IN the present study the technique known as microarray allowed for a detailed analysis of gene expression in the brains of *T. gondii*-infected, C57Bl6/J mice maintained on one of these two diets. The microarray (Agilent Oligo Microarray, Whole Mouse Genome) allowed for comparisons between levels of immunologically relevant gene transcripts in the brains of both infected mice and non-infected mice on each diet (4 groups; 4 mice per group). In this analysis, I focused on the following genes: TNF sf, IFN-γ, various interleukins, TGF-β, and Nos. Transcripts for the following genes were up-regulated (fold change>1; $p< 0.05$) in infected, antioxidant-supplemented-diet mice as compared to non-infected, antioxidant-supplemented diet mice: TNF sf 10 and TNF sf 13b, IFN-γ, IL-1β, 1L-18, and TGF-βi . The following genes were up-regulated in infected, antioxidant-deficient-diet mice: TNF sf 10, IFN-γ, and TGF-βi. Overall, there were higher levels of pro-inflammatory gene transcripts and lower levels of immuno-regulatory gene transcripts of Th 1 suppressing cytokines in infected mice maintained on an antioxidant-deficient diet. These results supported the hypothesis that the increased pathology observed in *T. gondii*-infected mice maintained on an antioxidant diet is associated with elevated expression of genes that promote harmful pro-inflammatory responses leading to increased pathology. Support of the National Institutes of Health and the National Center for Research Resources Grant P20 RR16481 and WKU Honors Program are gratefully acknowledged.

Introduction

***Toxoplasma gondii*: Origin, Life Cycle, Disease, and Treatment.**

In 1908, Nicolle and Manceaux (North Africa) and Splendore (Brazil) independently discovered *Toxoplasma gondii*, an obligate intracellular protozoan (Black and Boothroyd, 2000). Toxoplasmosis, caused by *T. gondii,* occurs in humans who are either immuno-competent or immuno-compromised (HIV/AIDS patients); both types of hosts have the potential for vertical infection across the placenta of a pregnant woman to her fetus. The disease is asyptomtomatic in immuno-competent individuals. Black and Boothroyd described the life cycle of this parasite and the macroscopic course of its contraction and its influences upon its hosts. The parasite infects and replicates within any nucleated mammalian or avian cell and divides its life cycle between feline (sexual stage) and nonfeline (asexual) infections. *T. gondii* infection is divided into acute and chronic phases depending on the parasite stage observed. The acute phase occurs when the tachyzoite stage, a rapidly growing form of the parasite, replicates within a cell until lysis occurs and the free-swimming tachyzoites infect neighboring cells. Seven to ten days post infection the disease moves toward the chronic stage as tachyzoites differentiate into bradyzoites and form tissue cysts.

The infection is acquired either through ingestion of tissue cysts in undercooked muscle tissue from intermediate hosts like cattle and pigs, or by accidental ingestion of sporulated oocysts present within contaminated cat feces. After ingesting the infected meat, or oocyst, the cysts rupture in the digestive tract, releasing the bradyzoites or sporozoites respectively (Black and Boothroyd, 2000). The parasites attack the lumen of the intestinal epithelium. Figure 1 displays a picture of the parasite's life cycle (Dubey, 1986).

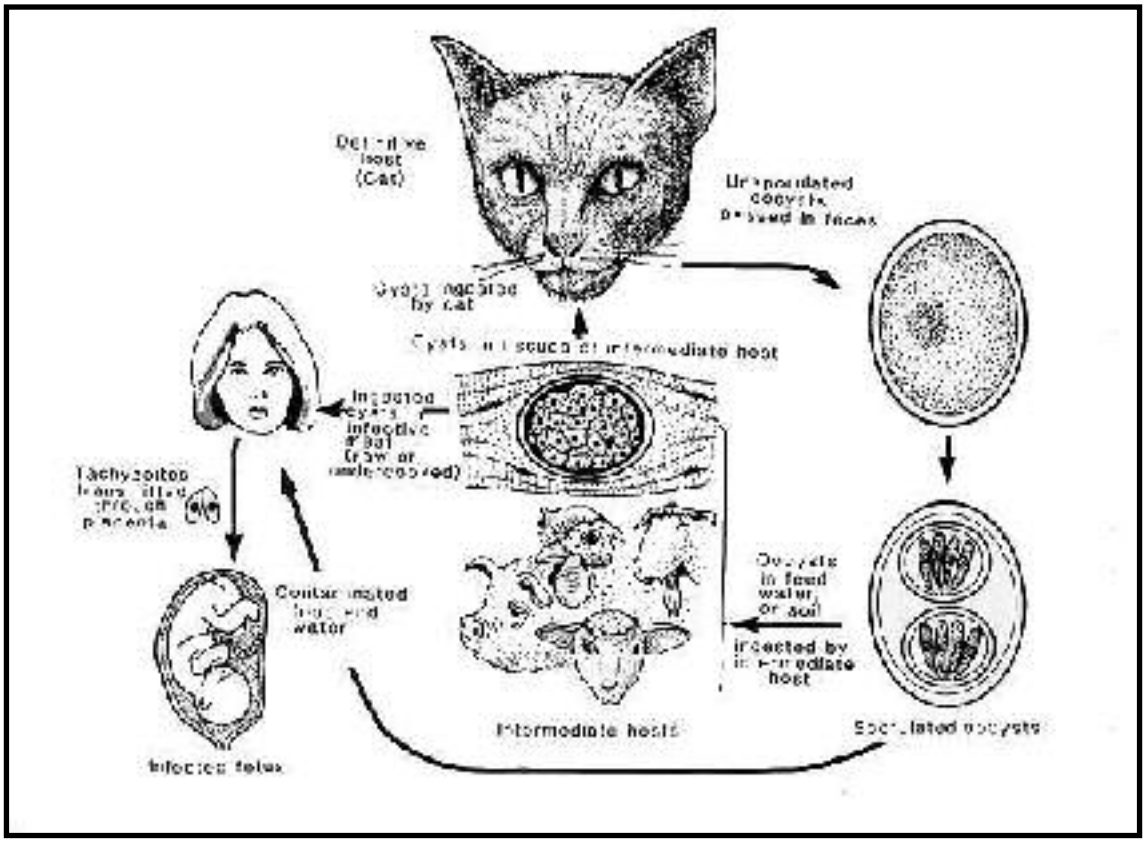

Figure 1. Life Cycle of *Toxoplasma gondii*
Source: J. P. Dubey
http://gsbs.utmb.edu/microbook/ch084.htm

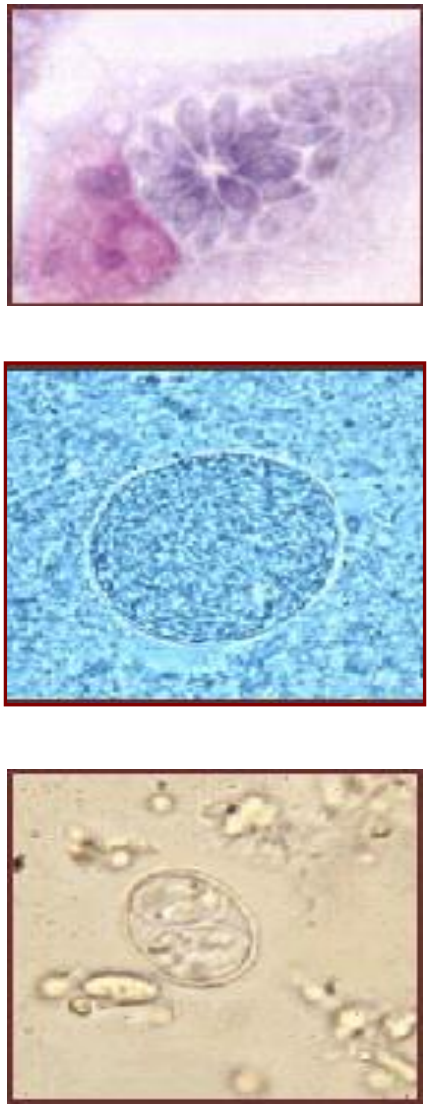

Figure 2. Different Stages of *T. gondii*. Provided from the National Institute of Infectious Disease in Japan. The pictures from top to bottom: a tachyzoites, tissue cyst of bradyzoites, and oocysts.

The host's immune response limits the replication of the intracellular stages and forces the parasite to encyst in the host's central nervous system and muscle tissue. Cysts remain viable for the life of the host. Recrudescence of the infection can occur.

The infection can cause serious health problems for those with compromised immune systems. Normally, rapid progression of the disease in the tachyzoite stage is combated and held in check by the type 1 (Th 1) immune response of an immuno-competent individual (Black and Boothroyd, 2000). Immuno-compromised patients endure symptoms including fever, enlarged neck lymph nodes, muscle pain, encephalitis, and eye inflammation. Vertical transmission of the infection to the fetus of an infected pregnant normal or immuno-compromised mother occurs during the acute phase of infection. Successful pregnancy is associated with the suppression of Th 1 cytokines to induce maternal tolerance towards the fetus, including the suppression of local inflammatory responses. This phenomenon contributes to the transplacental transfer of the parasite during pregnancy. The endangered fetus faces the possibility of blindness, mental retardation, and possible abortion or stillbirth. *T. gondii* may also cause intracerebral focal lesions that develop into Toxoplasmic Encephalitis (TE), a potentially fatal condition.

Drug therapy has been the primary treatment against this infection. Sulfonamide and pyrimethamine have been used against the tachyzoite stage, but they do not affect the chronic encysted bradyzoite stage (Black and Boothroyd, 2000). Unfortunately, these drugs have toxic side effects. Scientists today are looking into ways to combat *T. gondii* infection without risking the patient's quality of health and life. Current research has contributed to the understanding of the interaction between *T. gondii* and the host's immune system response. From these research efforts, better methods may be developed to assist the immune system in combating the disease more effectively in the near future.

Types of Immune Reactions and Cytokines.

Cytokines are low-molecular-weight proteins or glycoproteins secreted by the immune cells for the regulation of other immune's effector cells' development and function in the response to stimuli (Goldsby et al., 2003). In immuno-competent hosts, the parasite elicits a type 1 response (Th 1) characterized by the secretion of interferon-gamma (IFN-γ) and interleukin-2 (IL-2) from T helper lymphocytes. The Th 1 response promotes cell-mediated immunity. The key response involves the macrophage cells (MΦs), cytotoxic T lymphocytes, natural killer (NK) cells, and many cellular secreted cytokines. Some of the Th 1-related cytokines include the following: IL-12, IL-18, and Tumor Necrosis Factor (TNF)-β and -α. Some Th 2 cytokines include: IL-4, IL-5, and IL-10.

Some of the Th 1 and Th2 cytokines exhibit a cross-regulatory relationship. IL-10 (Th 2) down-regulates the secretion of macrophage cytokines (Th 1) including, IL-12, IL-1, and TNF-α. IL-10 can also affect the MΦ's function. For example, it reduces the production of reactive oxygen molecule intermediates and nitric oxide (NO) by MΦ cells. Importantly, the suppression of cytokine production in MΦ cells also indirectly results in the down-regulation of Th 1 cells and their cytokine secretions.

IFN-γ.

In immuno-compromised, *T. gondii*-infected hosts, cells other than T lymphocytes were investigated to determine other possible cellular origins of IFN-γ secretions (Suzuki et al., 2005). IFN-γ is known to be secreted by of T lymphocytes and NK cells in the brain during acute infection. Athymic nude and severe combined immunodeficient (SCID) mice lack T and B cells.

The deficiency of T and B cells in this mouse model during *T. gondii* infection provided an opportunity to determine if there were other sources of IFN-γ secretions. The authors' data demonstrated that major portions of brain cells (45-60 %) were marked positively for IFN-γ expression. Most of these IFN-γ-positively-marked brain cells were found to be NK cells. Less than 5% of the 45–60 % was identified as MΦ cells (detected by CD11c cell surface markers). A population of CD11b-cells from infected animals produced large amounts of mRNA for IFN-γ when compared to CD11b cells from non-infected animals. Brain cells from infected SCID mice also tested positive for IFN-γ after treatment with anti-asialo-GM1 antibody to eliminate NK cells. Suzuki and coworkers (2005) concluded that in infected, $NK^{-/-}$ mouse knockouts, $CD11b^{+}$ $CD45^{low}$ microglia cells and $CD11b^{+}$ $CD45^{high}$ blood–derived MΦ cells were the major non-T, non-NK cells that expressed INF-γ. The production of IFN-γ by these cells resulted from the induction of MHC (major histocompatibility complex) Class I and II expression in microglia and astrocytes. The MHC proteins are responsible for presenting antigenic peptides to T helper lymphocytes. The Class I MHC molecules are expressed on the surface of nearly all nucleated cells to interact with T cytotoxic lymphocytes. The Class II MHC molecules permit the macrophages, DC, and B cells to function as APCs (antigen presenting cells) to the T helper lymphocytes. In Suzuki and coworkers' study, these $CD11b^{+}$ $CD45^{low}$ microglia cells and $CD11b^{+}$ $CD45^{high}$ blood–derived MΦ cells then functioned as non-professional APCs to T helper lymphocytes in the brains, further activating other pro-inflammatory Th 1 cytokines in addition to IFN-γ.

IFN-γ versus IL–10 Effects on Nitric Oxide Production.

Rozenfeld and co-workers (2003) investigated neuroprotective solutions for the parasite-induced CNS inflammation triggered by immunoregulatory mediators. Nitric oxide (NO), a major toxic metabolite secreted by IFN-γ–activated microglia, may cause neurodegeneration during *T. gondii* infection. Various soluble factors [prostaglandin E_2 (PGE_2), interleukin-10 (IL-10), and RP–8–Br cyclic AMP (cAMP)], were investigated for their ability to reduce NO production in *T. gondii*-infected astrocytes and IFN-γ–activated microglia cells. IFN-γ is a major cytokine involved in the host's protective immune response to *T. gondii* infection. Specifically, IFN-γ is known to prevent the spread of tachyzoites in the acute phase, and to promote the conversion of tachyzoites into encysted bradyzoites. Rozenfeld and coworkers (2003) noted that encysted bradyzoites were not destroyed by the IFN-γ-activated phagocytic MΦs or cytotoxic $CD8^+$ T lymphocytes (via apoptosis = cell death). Although IFN-γ production was shown to result in NO production, this response did not appear to result in neuronal or other tissue damage in immuno-competent hosts. The authors suggested the possibility of additional mechanism(s) for the prevention of neuronal degeneration caused by the NO production.

Rozenfeld and co-workers (2003) found that PGE, produced by monocytes, and cAMP, produced in microglia cells, suppressed Th 1 cytokine production and favored Th2 cytokines like IL-10 that are associated with increased susceptibility to infection. IL-10 inhibited the production of NO, other oxygenated derivatives, and pro–inflammatory cytokines (TNF-α, IL-1, IL-6). The secretion of IL-10 resulted in a negative regulation of Th 1 inflammatory response and the production of IFN-γ and NO. The authors hypothesized that this mechanism is protective of CNS homeostasis in an infected, immunocompetent host.

Fetal and Maternal Infection.

During pregnancy, the responses of the maternal immune system to infection by *T. gondii* are even more complex. Fetuses combating congenital toxoplasmosis may be at risk for ocular and neurological defects or even death. Successful pregnancy is associated with the suppression of Th 1 cytokines to induce maternal tolerance towards the fetus, including suppression of local inflammatory responses. The suppression of maternal Th 1 cytokine production may allow a window of opportunity for the maternal *T. gondii* infection's tachyzoite stage to proliferate across the placenta to the fetus (Abou-Bacar et al., 2004).

Abou-Bacar and coworkers (2004) investigated the role of NK cells and the production of IFN-γ from those NK cells during the immune response to primary congenital toxoplasmosis. The recombination/activating genes (RAG 1 and 2) and their proteins are essential for the rearrangement of other immunoglobulin genes during B cell development and T cell receptor (TCR) genes during T cell development. The NK knockout mice were used as the experimental group. Meanwhile, their controls were defined as wild type (WT) mice and RAG $^{-/-}$ mice, which lack T and B cells. As a result, the investigators were able to observe the effects on both maternal infection and fetal infection with and without the contribution from certain components of the immune system.

Surprisingly, the normal mice had a higher risk (63%) of fetal toxoplasmosis than RAG $^{-/-}$ knockout mice (25%). Risk was measured by increased INF-γ production by maternal NK cells, fetal NK cells, and spleen cells concurrent with only a decrease in maternal – not fetal – parasitemia (Abou-Bacar, 2004). The WT control mice were used as a standard for comparisons of both the rate of maternal infection and maternofetal transmission. In RAG$^{-/-}$ mice lacking NK cells, the rate of fetal infection and maternal infection increased. The absence of IFN-γ from

Figure 3. Girl with Hydrocephalus due to Congenital Toxoplasmosis.
Source: J. P. Dubey and C. P. Beattie (1988)
http://gsbs.utmb.edu/microbook/ch084.htm

infected $RAG^{-/-}$ knockouts and infected WT mice resulted in decreased rates of maternofetal transmission, and surprisingly helped further the maternal infection. These results suggested that IFN-γ has directly or indirectly increased the possibility of transmission across the placental barrier. However, the increased number of NK cells producing IFN-γ served some protective immunity against transmission to the fetus.

Pathogenesis of Murine Ocular Toxoplasmosis and IL-10.

IL-10 is a regulatory cytokine with anti-inflammatory and immunosuppressive properties (Goldsby et al., 2003). For example, it reduces IFN-γ and macrophage cytokine responses to acute intracellular infection, protecting the host from an excessive and potentially lethal Th 1 cytokine response resulting from immunopathology.

IL-10 also has been shown to play an important role in the pathogenesis of ocular toxoplasmosis (Lu, Huang, and Kasper, 2003). IL-10 is required for the restriction of ocular immunopathology. Lu and coworkers (2003) conducted a study in which the following mice were challenged with ocular infection: B6 and BALB/c WT, B6 transgenic mice with IL-10 expression under an IL-2 promoter, and BALB/c mice that lacked a functional IL-10 gene. Eye tissue was examined for necrosis and inflammation at different periods after infection (4 days up to 11 days).

The results of the study indicated that IL-10 knockout mice had an unchecked increase in the Th 1 response (Lu et al., 2003). This response included higher levels of IFN-γ and TNF-α leading to major tissue damage during the inflammatory response to tachyzoite proliferation and loss of control over intracerebral *T. gondii* leading to TE. The infection was best held in check by the transgenic mice with IL-2 promoters (Th 1). These mice experienced the least amount of

ocular pathology and no necrosis. Therefore, the authors concluded that IL-10 helps down regulate tissue pathology in both B6 and BALB/c mice in the protective immune responses against *T. gondii*.

Future Immunization against *T. gondii* Infection.

An interesting technique of murine immunization was developed using synthetic dendrite cell (DC)-derived exosomes pulsed with *T. gondii* antigen to prime a specific cellular and humoral Th 1-based immune response without exposure to the *T. gondii* cellular parasite (Aline et al., 2004). This immunization protocol successfully induced protection against the RH strain of *T. gondii* infection in the spleen cells of C57BL/6 mice. The authors detected early cellular immunity when APCs and DCs developed the antigen–specific response by switching the T helper cells to Th 1 lymphocytes (including CD 4^+ and $CD8^+$ T cells that will produce IFN-γ). IL-12 secreted by the DCs supported the Th 1 response by promoting IFN-γ production. DCs were found capable of secreting exosomes that contain the class I and II MHC molecules, and the exosomes were capable of fusion with the plasma membranes of other APCs.

Aline and coworkers' study (2004) was the first in which anti-tumor DC-derived exosomes pulsed with non-cellular *T. gondii* antigens were transferred into the intestines of mice, thereby inducing an optimized immune response against the chronic and acute stages of infection (2004). The investigators measured increased fluorescence levels of major exosomal components involved in the interactions between the DC exosomes and the APC and those interactions between target cells and T lymphocytes, including fluorescent tags for the following: MHC class I and II, CD80 (B7-1 costimulatory signal), CD86 (B7-2 costimulatory signal), CD32 (FcγRII receptor for IgG antibody), and CD 54 (Intercellular Adhesion Molecule-1 – ICAM-1 –

ligand for CD11a/CD18 or CD11b/CD18). The pulsed exosomes were not found in high concentrations within collected liquid supernants of non-homogenized mouse tissue. In other words, the host's organs and tissue absorbed the pulsed exosomes. Localization of the exosomes included the intestines, the cervical lymph nodes, and the spleen. A Western blot analysis recorded amounts of IgM and IgG from the intravenously treated, infected mice. The treated, infected mice experienced less mortality during the acute phase infection. Cytokine production of the Th 1 subset (including IL-2 and IFN-γ) was favored in treated mice over the Th 2 subset (including IL-10 and IL-5). Treated, infected mice also had less cyst formation than untreated infected mice. This study provided the evidence for very effective immunity against the chronic stage of infection through the application of non-cellular, synthetic exosomes.

T. gondii's Surface Proteins' Number, Evolution, and Developmental Expression.

T. gondii has a wide range of hosts that it can infect. The surface of tachyzoite and bradyzoite stages contains glycosylphosphatidylinositol-linked proteins structurally related to the highly immunogenic surface antigen SAG1 (Jung, Lee, and Griggs, 2004). These surface antigens belong to the SRS (SAG1-related sequences) super family of proteins, which serve as attachment molecules to the host cells and activate host immunity in favor of the parasite's virulence.

According to the authors, little is known about the specific mechanisms by which the proteins promote their function, but Jung and co-workers (2004) researched the number, evolution, and developmental expression of SRS genes. They discovered 114 new sequences of SRS proteins scattered among the type II ME-49 *T. gondii* strain's genome. They suggested that the surface antigens might be important in determining which host ligands the SRS antigen is

capable of binding to, thus explaining why the parasite has such a wide spectrum of host cell types. Future research will reveal more about the proteins' varied functions and mechanisms involved with the binding of *T. gondii* to different cell types. With more knowledge about the binding mechanism, scientists may be able to design a treatment that will disable the parasite's binding ability. Clearly, more research is needed in this area.

Previous Studies on the Effects of Antioxidant Diet in a Murine Model of Toxoplasmosis.

Previous studies performed within the lab of Dr. Cheryl Davis of Western Kentucky University provided evidence that dietary supplementation with the antioxidants vitamin E and selenium is harmful during *T. gondii* infection in both Swiss Webster and C57BL/6 mice. However, a diet lacking in these antioxidants resulted in reduced tissue pathology, including decreased counts of tissue cysts and a reduction in weight loss during infection (McCarthy and Davis, 2003). This result was unexpected considering the large body of evidence suggesting that antioxidant supplementation is beneficial during other parasite infections; for example, Chagas' disease is caused by a parasite named *Trypanosoma cruzi*. Previous studies in our lab clearly demonstrated that antioxidant supplementation assists the body's immune response against *T. cruzi* infection (Davis et al., 1998). It is also interesting to speculate about the implications for HIV-infected patients who are routinely supplemented with antioxidants to reduce the oxidative stress that contributes to the HIV viral replication (Baruchel and Wainberg, 1992). Another important study could look into the effects of antioxidant supplemented and deficient diet treatments in individuals co-infected with HIV and *T. gondii*.

Antioxidants and Health.

Antioxidants can prevent damage to a normal cell's membrane and the cellular DNA caused by unstable molecules called free radicals (National Cancer Institute, 2004). Free radicals are elements in which bonds have abnormally split, resulting in an unpaired electron in the element's outer electron valence shell (Wade, 2003). This splitting (initiation step) of radical formation is caused by heat, light, peroxides, and a number of environmental contributors like cigarette smoke, pollution, and radiation. The normal oxygen molecule has a strong tendency to lose electrons or oxidize. This process in the body often results in two unstable oxygen radicals, each containing an unpaired electron. Unstable free radicals react very quickly to capture an electron from the nearest compound available. The process becomes carcinogenic as the "attacked" compound loses its electron to the unstable radical oxygen, becoming a free radical itself. A chain reaction forms, leading to the disruption of a living cell.

Normal metabolic activity results in the production of free radicals. The radicals are neutralized with pre-consumed and naturally made antioxidants; however, excessive cellular damage occurs when the production of free radicals increases beyond the body's natural ability to correct in response. Free-radical damage tends to accumulate with age (National Cancer Institute, 2004). It is important to maintain a diet of fruits and vegetables containing antioxidants and the pre-cursor molecules. Important dietary antioxidants include beta-carotene, lycopene, vitamins C, E, and A, and other substances. Selenium (Se) is a mineral found in meats and breads; Se also serves as a cofactor to antioxidant enzymes. Se is not manufactured in the body, so it must be obtained through the diet. Vitamin E (alpha-tocopherol) is found in almonds; in many oils, including wheat germ, safflower, corn and soybean oils; and found in mangos, nuts,

broccoli, and other foods. Vitamin E is lipid soluble, making it one of the most effective antioxidants in the protection against cell membrane damage.

Antioxidants protect against free-radical damage by policing the chain reaction of electron stealing (Wade, 2003). They neutralize the free radicals by contributing an electron without becoming a radical themselves. The antioxidants are stable in both forms, thereby preventing cell and tissue damage leading to pathology and disease.

Goal of Present Study.

In this investigation, a new technique known as Microarray was employed to evaluate the specific impact of *T. gondii*-infection on gene expression in brains of mice treated with an antioxidant-supplemented diet or a diet deficient in antioxidants. The levels of several well-known, immunologically relevant gene transcripts were analyzed here to depict a primary picture of Th1 and Th2 responses. Years of information pertaining to the global gene expression during murine *T. gondii* infection are obtainable from the microarray data. It is the long-term goal of our laboratory to determine the impact of diet on global gene expression in the brains of infected and non-infected mice using the microarray data. In addition, the lab plans to identify the major genes that are differentially expressed, determine their function, and discover the molecular pathways in which these genes participate. It is our hope that the information generated by these studies will eventually lead to improved treatment options for *T. gondii* infection that do not decrease the patient's quality of life.

Based on the results of previous studies at Western Kentucky University, severe toxoplasmic encephalitis was exhibited in mice receiving antioxidant supplementation. Therefore, it was hypothesized that the microarray analysis would provide evidence for elevated

expression of genes that promote harmful pro-inflammatory responses in mice receiving antioxidant-supplemented diets.

Materials and Methods

Mouse Model.

Sixteen female C57Bl6/J mice (Jackson Laboratories, Bar Harbor, Maine), five weeks of age at the beginning of the study, were divided into four groups. This particular mouse strain is highly susceptible to the development of toxoplasmic encephalitis following infection with the ME-49 strain of *T. gondii*. The model is also beneficial because its entire genome has already been sequenced.

Experimental Groups.

Sixteen C57Bl6/J mice were maintained on one of two diets. Eight mice were supplied with diH_2O as drinking water and a diet containing 0 I.U. /kg vitamin E and 0 ppm selenium. Eight mice were given diH_2O containing 8 ppm sodium selenate as drinking water and a diet containing 400 I.U. /kg vitamin E. Diets were specially formulated by Purine Mills Inc. Mice were maintained on the experimental diets for four weeks. Four mice out of each diet treatment group were then infected intraperitoneally with 5×10^3 tachyzoites of *T. gondii*.

Parasite Model.

The parasites (ME-49 strain of *T. gondii*) were maintained in CV-1 cells (African Green Monkey Kidney cells) as a source of tachyzoites (provided by Dr. David Lindsay of Virginia Tech). The tachyzoites were harvested from culture supernants. Parasite counts were performed using a hemacytometer.

RNA Isolation.

The two groups of four infected mice (four infected mice on deficient diet and four infected mice on antioxidant-supplemented diet) and the two diet groups of four non-infected control mice were maintained for two additional weeks following infection. At 2 weeks post infection, all sixteen mice were euthanized. Under aseptic conditions, their brains were removed, placed into cryovials, and flash frozen in liquid nitrogen. The RNA fractions were isolated using an RNeasy Lipid Midi Kit (Qiagen) according to manufacturer's instructions. RNA concentrations were determined spectrophotometrically, and agarose gels confirmed the purity of RNA. Prior to microarray analysis, RNA levels were quantified, and RNA integrity was confirmed using an Agilent 2100 Bioanalyzer.

Microarray Analysis.

Pure RNA samples from all sixteen mice (four groups of four biological replicates or mice) were taken to the Microarray facility at the University of Louisville Health Sciences Center. Agilent Oligo Microarray (Whole Mouse Genome) allowed for the simultaneous analysis of approximately 44,000 genes. My analysis focused specifically upon the following genes: IFN-γ gene (interferon), various interleukin genes (IL), TNF sf genes (tumor necrosis factor super family), TGF-β genes (transforming growth factor-beta), and Nos genes (nitric oxide synthase). One microarray chip was used for each mouse. Differences in levels of gene expression (measured as spot intensity) between two groups were analyzed by the T test, with the statistical significance defined as $p < 0.05$.

Results

In this analysis, we focused specifically upon the following genes: IFN-γ gene (interferon), various interleukin genes (IL), TNF sf genes (tumor necrosis factor super family), TGF-β genes (transforming growth factor-beta), and Nos genes (nitric oxide synthase). All reported values represent significant ($p < 0.05$) differences in the levels of gene expression between two groups.

Graphs were constructed based on the immunologically relevant gene transcripts (x-axis) and the fold change or difference in the gene transcripts' abundance (y-axis) between two experimental groups. The significant ($p < 0.05$), up-regulated gene transcripts were defined as those with fold changes above one. The significant gene transcripts with fold changes at the value of one experienced no change in their gene transcript regulation. All the significant gene transcripts with fold changes that are less than one were down-regulated.

Table 1 displays the total RNA quantified and used from each mouse in all four groups. The lettered-numbers represent the mouse used within the group. The B and G groups were non-infected (F and H infected). The F and B groups were given a deficient diet (G and H an antioxidant diet). The Abs stands for spectrophotometric absorbance. The Concentration of RNA measured from each isolation is recorded in units of nanograms per microliters. The Total column indicates the total RNA concentration of each sample in micrograms. The agarose gel, shown in Figure 4, confirmed the high yield and purity of all sixteen RNA samples displayed in Figure 3.

Table 2 includes a list of all the investigated gene transcripts. This table presents the known sources and activities of the gene transcripts. This information was used as a tool to help

	Abs	Conc (ng/µl)	Total (µg)
B1	0.63	1254	69
B2	0.46	928	51
B3	0.67	1341	73.8
B4	0.78	1570	86.3
F1	0.37	736	40.5
F2	0.4	793	43.6
F3	0.48	958	52.7
F4	0.53	1069	58.8
G1	0.5	1006	55.3
G2	0.41	830	45.6
G3	0.56	1111	61.1
G5	0.48	963	53
H1	0.56	1112	61.2
H2	0.48	969	53.3
H3	0.61	1210	66.6
H5	0.29	577	31.7

Table 1. Total RNA from Mice Brain Tissue. This table contains the four groups of four mice and corresponding RNA concentrations.

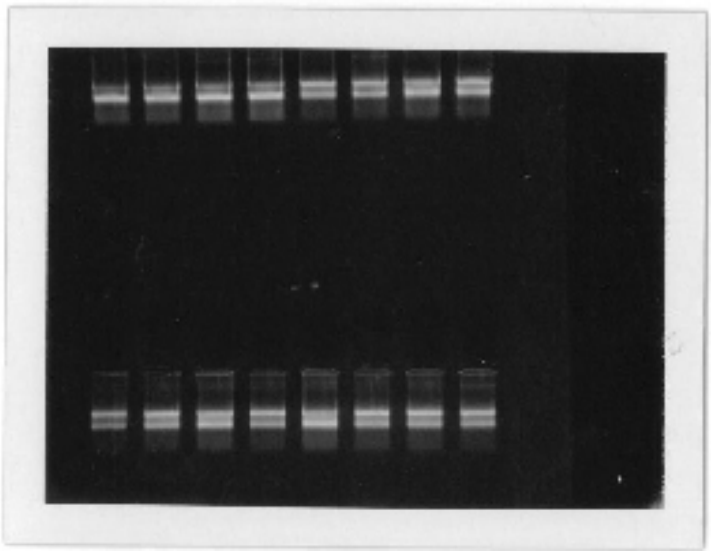

Figure 4. Gel Electrophoresis. A picture of the agarose gel of all RNA samples confirming purity of RNA isolation.

in the interpretation of the data presented in each graph.

Figure 5 shows the comparison between levels of gene expression in the brains of infected mice maintained on antioxidant-deficient diet and the levels of gene expression in the brains of non-infected, control mice on the same diet. Genes that were up-regulated (fold change>1; $p< 0.05$) in the brains of infected, antioxidant-deficient diet mice as compared to non-infected, antioxidant-deficient diet mice were as follows: TNF sf 10, IFN-γ, and TGF-βi. The following genes were down-regulated (fold change<1; $p< 0.05$) in the same comparison: IL-2 and TGF-β1.

Next, Figure 6 exhibits the graphical comparison between levels of gene expression in the brains of infected, antioxidant-supplemented-diet mice, as compared to the levels of gene expression in the brains of the non-infected mice maintained on antioxidant-supplemented diet.

	Cytokine	Sources	Activity
TNF super family	TNF-α	Monocytes, MΦs, T lymphocytes (cells), and fibroblast	Inhibits growth of a number of cell types; affects tissue remodeling, wound repair, development, and hematopoiesis. Pro-inflammatory contributor. Exert suppressive effects on the expansion of certain immune-cell populations.
	TNF-β	Activated T cells; B cells	Mediator of inflammation and immune function. Affects healing.
	IFN-γ	$CD4^+$ and $CD8^+$ T cells, NK cells	Affects activation, growth, and differentiation of T cells, B cells, NK cells and MΦs. Up-regulates MHC expression on APCs. Signature cytokine of the Th 1 differentiation. Involved in attracting and activating MΦs during inflammation response. Weak anti-viral and anti-proliferative activities.
Interleukins	IL-1	Monocytes, MΦs, DCs, T and B cells, NK cells, and vascular epithelium, fibroblasts, and some smooth muscle cells	Inducer of fever, local coagulation, vascular permeability, the acute phase response, chemotactic factors and stimulation of neutrophil production. Increases the expression of adhesion molecules on vascular endothelial cells.
	IL-2 or T cell Growth Factor	T cells	Stimulates growth and differentiation of T cells, B cells, and NK cells.
	IL-7	Bone marrow stromal cells, thymic stromal cells, and spleen cells	Growth factor for T and B cell progenitors.
	IL-13	Activated T cells, mast cells, and NK cells	Role in the Th 2 responses by suppressing inflammatory responses and/or up- regulating synthesis of IgE.
	IL-16	T cells	Stimulates migration of $CD4^+$ T cells, $CD4^+$ monocytes, and eosinophils. Binding of IL-16 by CD4 inhibits HIV infection of $CD4^+$ cells.
	IL-17	$CD4^+$ T cells	Supports hematopoiesis indirectly by stimulating cytokine production by epithelial, endothelial, and fibroblastic stromal cells. Enhances the expression of ICAM-1, thus making cells more adhesive.
	IL-18	Cells of monocytic lineage and DCs	Promotes differentiation of Th 1 subset of helper T cells. Induces IFN-γ production of T cells and enhances NK cell cytotoxicity.
	IL-19	LPS-stimulated monocytes and other likely undocumented sources	Newly discovered member of the IL-10 family of cytokines. Activity under investigation. IL-10: Stimulates or enhances proliferation of B cells, thymocytes, and mast cells. In cooperation with TGF-β; stimulates IgA synthesis and secretion by B cells; antagonizes generation of the Th 1 subset of helper T cells.
	IL-22	Unknown	Similar to IL-12 functions: inducing differentiation of Th 1 subset helper T cells. Induces IFN-γ production of T cells and enhances NK cell cytotoxicity
Transforming Growth Factor β	TGF-β	Nucleated cell types and platelets	Inhibits growth of a number of cell types; affects tissue remodeling, wound repair, development, and hematopoiesis. Exert suppressive effect on the expansion of Th 1 immune responses.
Nitric Oxide Synthetase	NOS 2 and 3	MΦs and neutrophils	Nitric oxide synthetase is important to anti-bacterial, anti-fungal, anti-parasitic, and anti-protozoan responses resulting in NO production.

Table 2. List of Immunologically Relevant Gene Transcripts and Their Activities.
Information obtained by tables from appendix II and page 40 in Goldsby et al. text (2003).

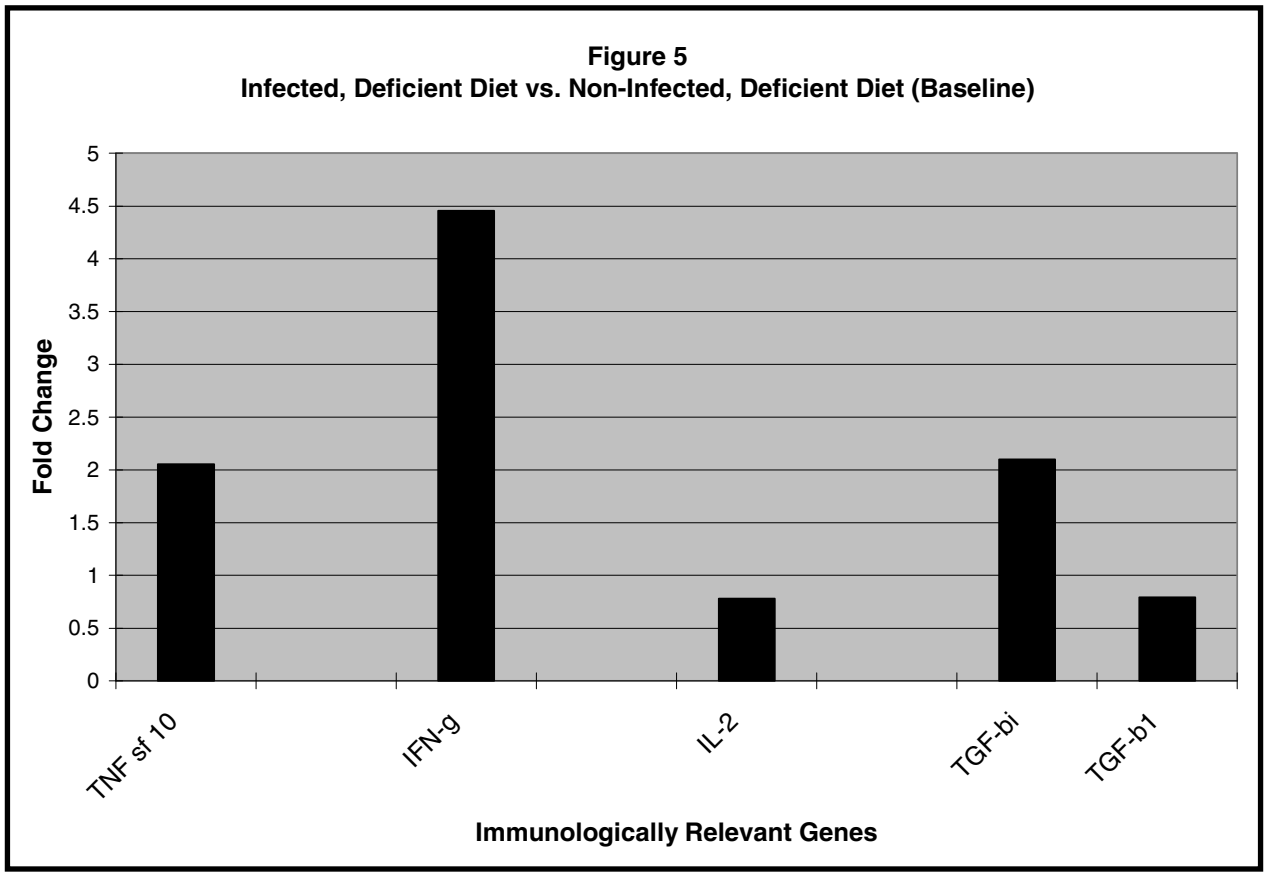

Figure 5. Infected, Deficient Diet vs. Non-Infected, Deficient Diet (Baseline). Graph shows the fold change values for the significant ($p<0.05$) gene transcripts.

In Figure 6, the following genes were highly up-regulated (fold change>1; $p< 0.05$) in infected mice: TNF sf 10 and TNF sf 13b, IFN-γ, IL-1β, 1L-18, and TGF-β induced. IL-13 and IL-17F gene transcripts were down-regulated (fold change<1; $p< 0.05$) in the same comparison.

Figure 7 displays the two deficient-diet groups' comparison (from Fig. 5) in black and the

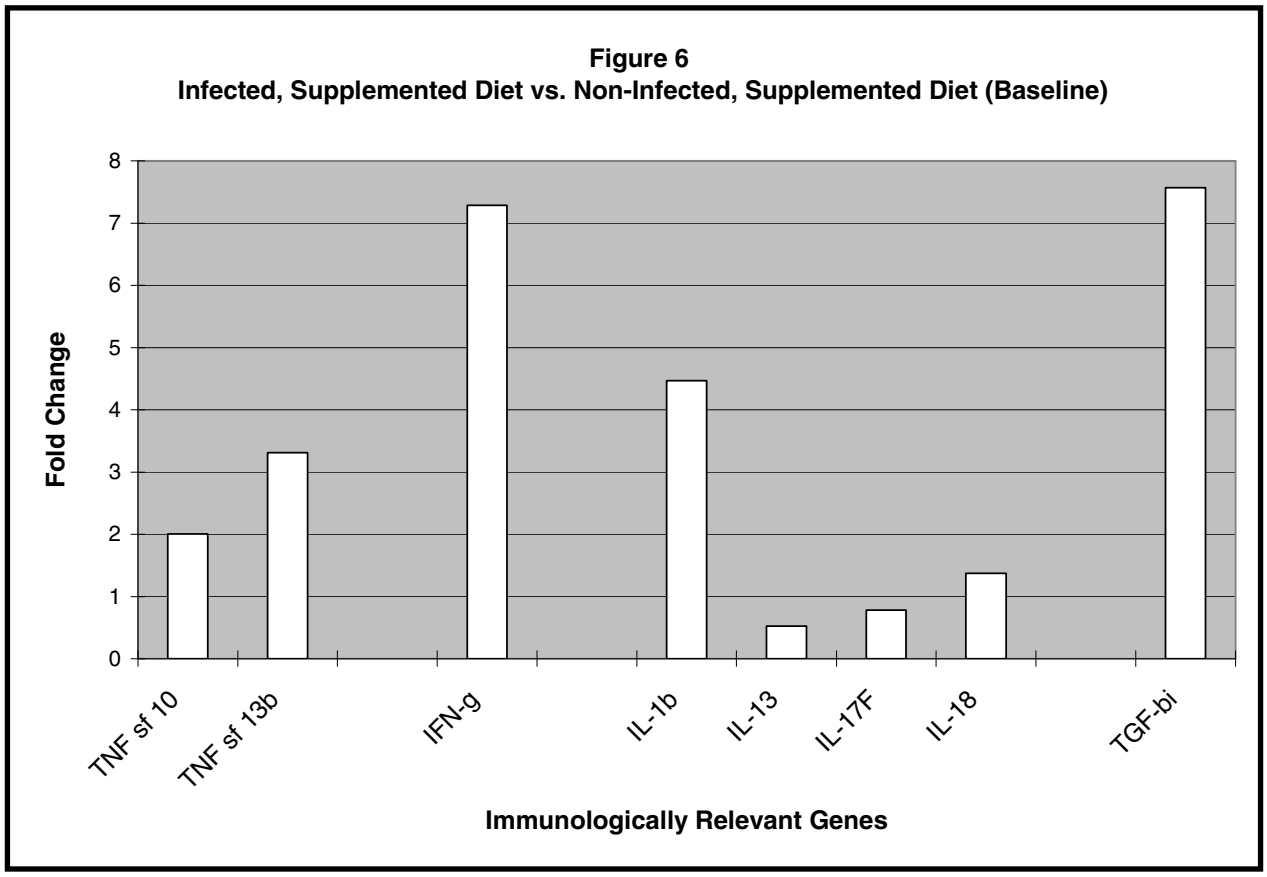

Figure 6. Infected, Supplemented Diet vs. Non-Infected, Supplemented Diet (Baseline). Graph shows the fold change values for the significant ($p<0.05$) gene transcripts.

two supplemented-diet groups' comparison (from Fig. 6) in white. This graph was used to place both of the diets' comparisons on the same fold change scale.

Finally, Figure 8 displays differences in the levels of immunologically relevant gene expression between the brains of infected, antioxidant-supplemented-diet mice and the infected,

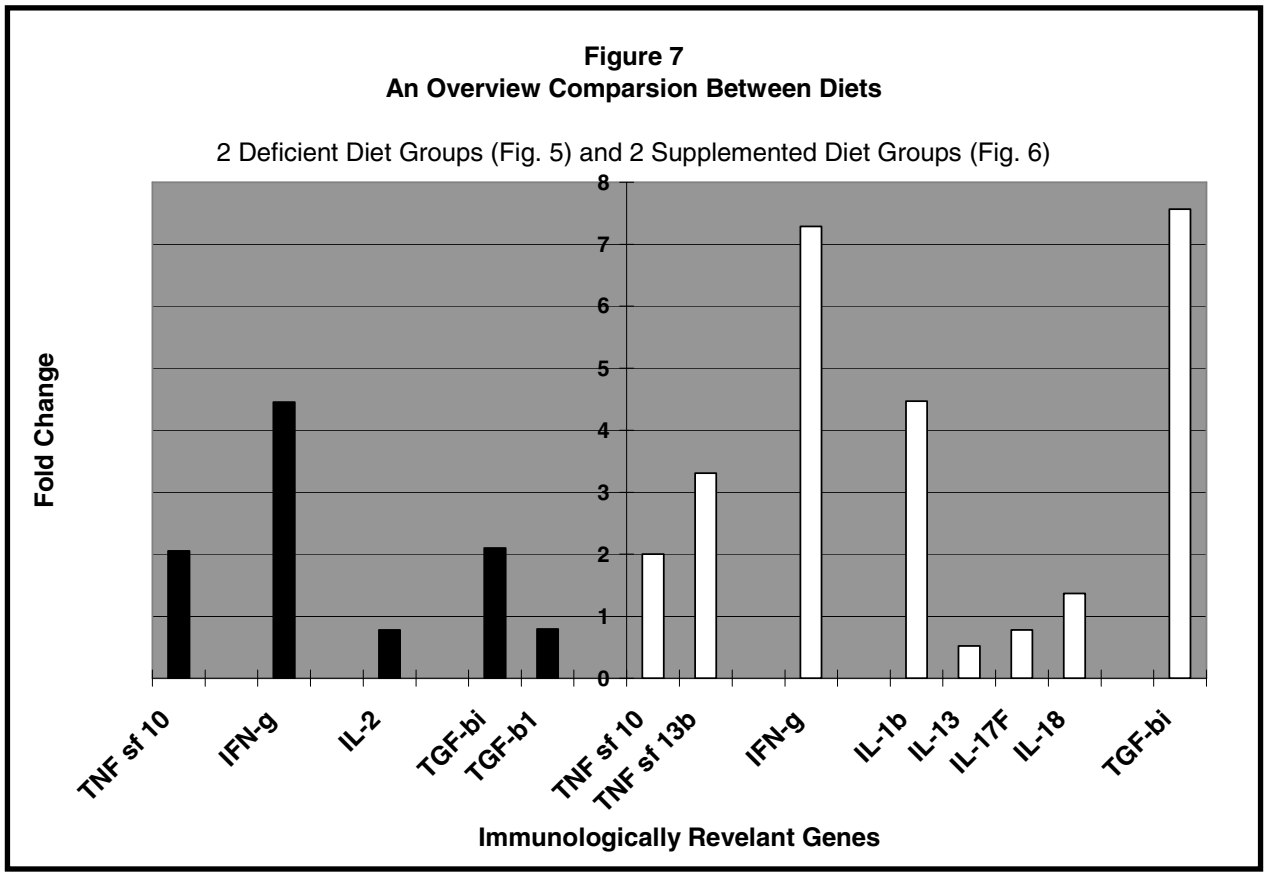

Figure 7. An Overview Comparison Between Diets. Two deficient-diet groups' comparison (Fig. 5) in black and two supplemented-diet groups' comparison (Fig. 6) in white have been placed on the same scale. Graph shows the fold change values for the significant ($p<0.05$) gene transcripts.

antioxidant-deficient-diet mice. The following genes were up-regulated (fold change>1, $p<0.05$) in infected, supplemented-diet mice when compared to their infected, antioxidant- deficient diet mice: TNF sf 13b, IFN-γ, IL-18, IL-22, IL-16, IL-19, TGF-β3, TGF-α, TGF-β induced, Nos 2,

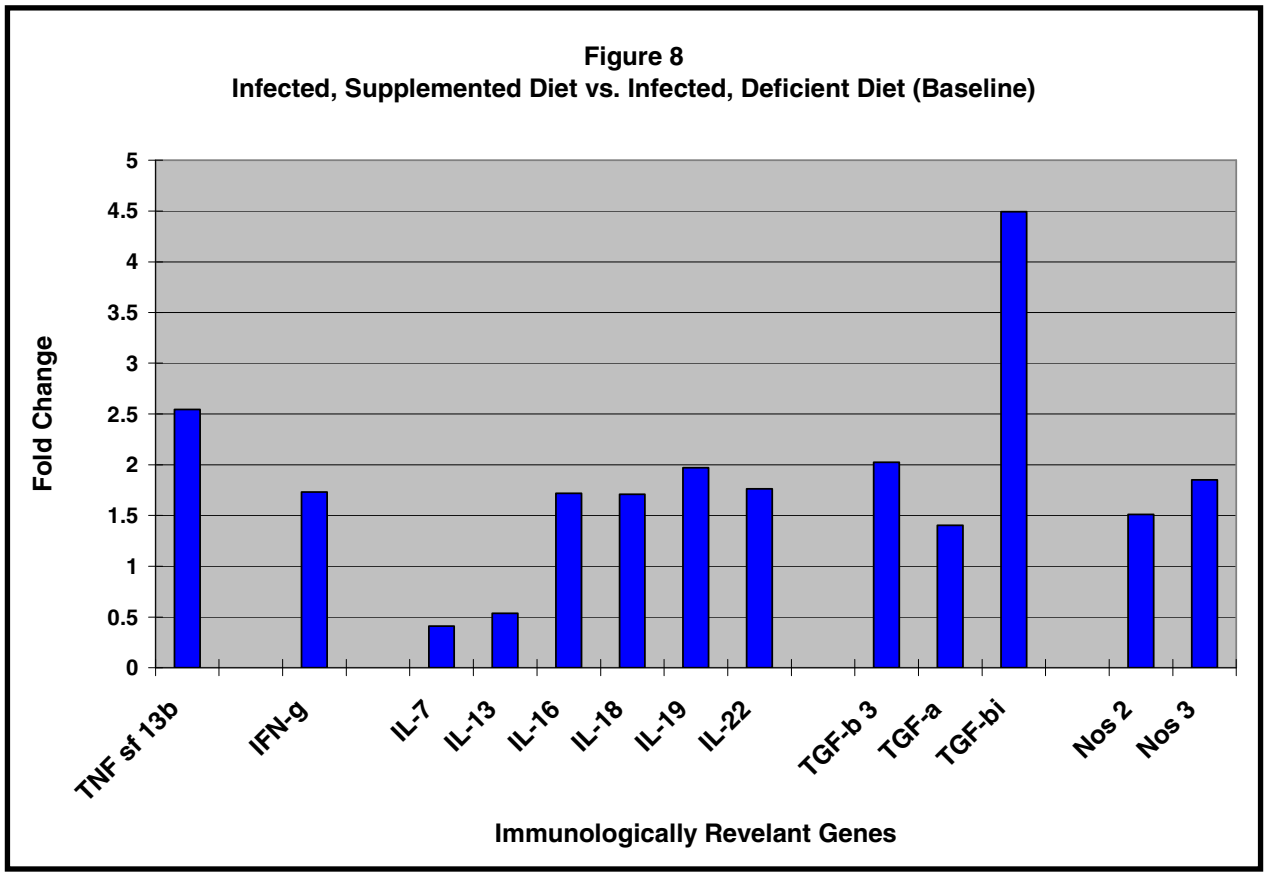

Figure 8. Infected, Supplemented Diet vs. Infected, Deficient Diet (Baseline). Graph shows the fold change values for the significant ($p<0.05$) gene transcripts.

and Nos 3. The IL-13 and IL-7 gene transcripts were down-regulated (fold change<1; $p< 0.05$) in the same comparison.

Discussion

AIDS patients with toxoplasmosis continue to face few treatment options that do not harm them while also disabling the parasite's proliferation. Normally, antioxidant dietary supplements are expected to reduce the chronic oxidative stress caused by the HIV and the parasite infection. However, studies in this lab have provided evidence that the addition of selenium and vitamin E to the normal diet negatively affects the outcome of infection (McCarthy and Davis, 2003). In mice deprived of selenium and vitamin E, there were actually fewer brain tissue cysts and reduced levels of inflammation as well as lower levels of Th1 cytokine production.

The microarray technique allows the simultaneous detection and measurement of mRNA levels corresponding to each gene in the mouse genome. The overall goal of the work in our lab is to determine the impact of diet on global gene expression in the brains of infected mice. We also intend to identify the major genes that are differentially expressed, determine their function, and discover the molecular pathways in which those genes participate. In this present study, only defined genes of immunological relevance were analyzed.

The results of this study suggest that mice maintained on a diet deficient in antioxidants (Figure 5) responded to infection by maintaining a controlled inflammatory response. Elevated levels of the pro-inflammatory cytokines TNF sf and IFN-γ were observed in the brains of infected mice; however, these levels were moderate and balanced by the increased expression of the regulatory cytokine, TGF-β, which increased the suppression of the expanding Th 1 type immune responses. Additionally, T cell-growth factor (IL-2) was down-regulated in infected, deficient mice.

In contrast, infected mice maintained on an antioxidant-supplemented diet showed even higher levels of expression of pro-inflammatory genes (IFN-γ, TNF sf, and IL-1b) and up-regulated levels of IL-18 Th 1 cytokine (see Figure 6). Table 2 describes each cytokine's functional role. In addition, IL-13 and IL-17F levels were actually down-regulated in the brains of these mice, allowing for an unchecked pro-inflammatory response in the infected, antioxidant-supplemented-diet mice.

Finally, Figure 8 demonstrates the degree of effectiveness of a deficient diet compared to an antioxidant-supplemented diet in infected mice. The infected, antioxidant-deficient-diet mice gene transcripts were used as the baseline for all of the fold changes in this graph's analysis. The following genes were up-regulated (fold change>1, $p<0.05$) in infected, supplemented-diet mice when compared to their infected, antioxidant- deficient diet mice: TNF sf 13b, IFN-γ, IL-18, IL-22, IL-16, IL-19, TGF-β3, TGF-α, TGF-β induced, Nos 2, and Nos 3. Of those up-regulated gene transcripts, the following contain pro-inflammatory functions and support Th 1 immune responses: TNF sf 13b, IFN-γ, IL-16, IL-18, and IL-22. The up-regulated Nos 2 and 3 gene transcript levels provide evidence for an increase in nitric oxide synthesis in the brains of mice given antioxidant-supplemented diets. In contrast, expression of the Th 2 cytokine, IL-19, was up-regulated, along with several highly up-regulated TGF family gene transcripts. This suggests an unsuccessful attempt to control the intense inflammation occurring in the brains of mice given an antioxidant-supplemented diet. Down-regulation of the gene transcripts for the Th 2 cytokines, IL-13 and IL-7, was also observed. Lower levels of IL-13 and IL-7 would result in a reduced suppression of inflammatory responses and fewer growth factors for T and B cell progenitors. Overall, the results displayed in Figure 8 provide evidence for a strong pro-

inflammatory response involving up-regulation in the genes for multiple inflammatory mediators.

In conclusion, the results of this microarray study do provide evidence for a damaging inflammatory response in the brains of *T. gondii*-infected mice maintained on an antioxidant-supplemented diet. In future studies, this analysis will be expanded to include the many other genes of immunological relevance that may be affected by the presence or absence of antioxidants in the diet.

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